Ann Toxicol Anal. 2011; 23(1): 7-14
© Société Française de Toxicologie Analytique 2011
DOI: 10.1051/ata/2011008

Available online at:
www.ata-journal.org

ANNALES DE TOXICOLOGIE ANALYTIQUE

## Original article / Article original

# Determination of the amphetamine-like designer drugs methcathinone and 4-methylmethcathinone in urine by LC-MS/MS

## *Détermination par LC-MS/MS des « drogues de synthèse » : la méthcathinone et la 4-méthylméthcathinone (substances analogues aux amphétamines) dans les urines*

Dirk Grueninger*, Ralf Englert

MVZ Clotten, Labor Dr. Englert, Dr. Raif und Kollegen, Bismarckallee 10, 79098 Freiburg, Germany

**Abstract –** **Purpose:** Beta-keto amphetamines are a new generation of designer drugs which have gained popularity among abusers in the last decade. A sensitive and reliable method for the diagnostics of individuals who abuse these substances is very important, especially in the case of overdose or concomitant use during a drug substitution therapy. **Methods:** We developed a quantitative HPLC-tandem mass spectrometry method for the determination of methcathinone and 4-methylmethcathinone in urine, based on an easy liquid-liquid extraction technique coupled to fast reversed phase chromatography. **Results:** For both analytes we obtained a linearity range from 0.5 to at least 2000 ng/mL. In different urinary matrices the accuracy for 4-methylmethcathinone was within 9% and for methcathinone within 17% recovery of the target concentration. The repeatability and the intermediate precision of 50 different test series were between 2 and 14%. The limit of quantification was established to be 2 ng/mL for methcathione and 0.8 ng/mL for 4-methylmethcathinone. **Conclusions:** The described HPLC-tandem mass spectrometry method is reliable and robust and has the advantage of accurate selectivity, avoiding wrong positive results. It is convenient for clinical laboratories involved in the control of drug substitution therapy or the confirmation of drug consumption.

**Key words:** Beta-keto amphetamines, designer drugs, liquid chromatography-tandem mass spectrometry, methcathinone, ephedrone, 4-methylmethcathinone, mephedrone

**Résumé –** **Objectif :** Les beta-kéto-amphétamines sont une nouvelle génération de drogues de synthèse dont l'usage s'est répandu chez les toxicomanes au cours des dix dernières années. Une méthode précise et fiable de dépistage chez les personnes susceptibles d'utiliser ces substances est importante, dans le cas de surdose ou d'utilisation concomitante pendant un traitement de substitution. **Méthodes :** Nous avons développé une méthode par CLHP couplée à la spectrométrie de masse en tandem pour le dosage de la méthcathinone et de la 4-méthylméthcathinone dans l'urine, qui repose sur une technique d'extraction liquide-liquide simple couplée à une chromatographie rapide à phase inverse. **Résultats :** Pour les deux substances à doser la méthode est linéaire de 0,5 à au moins 2000 ng/mL. Dans différentes matrices urinaires, la précision pour la 4-méthylméthcathinone était d'environ 9 %, et pour la méthcathinone d'environ 17 % de récupération de concentration cible. La répétabilité et la précision intermédiaire sur 50 dosages différents s'échelonnaient entre 2 et 14 %. La limite de quantification s'est établie à 2 ng/mL pour la méthcathinone et à 0,8 ng/mL pour la 4-méthylméthcathinone. **Conclusions :** Cette méthode de dosage de la méthcathinone et de la 4-méthylméthcathinone dans l'urine par CLHP couplée à la spectrométrie de masse en tandem est fiable et spécifique, évitant les faux positifs. Elle est adaptée aux laboratoires assurant les contrôles dans le cadre de traitements de substitution ou le dépistage de l'usage de stupéfiants.

**Mots clés :** Beta-kéto-amphétamines, drogues de synthèse, tandem chromatographie liquide/spectrométrie de masse, méthcathinone, éphedrone, 4-méthylméthcathinone, méphedrone

Received 29 September 2010, accepted after revision 17 February 2011
Published online 7 April 2011

* Correspondence: Dirk Grueninger, d.grueninger@mvz-clotten.de

## 1 Introduction

Methcathinone (MC, ephedrone) and 4-methylmethcathinone (4-MMC, mephedrone) are beta-keto amphetamines which belong to a new generation of designer drugs (Fig. 1).

During the last decade, these drugs of the cathinone type have infiltrated rapidly into the drug market [1, 2]. The identification of beta-keto amphetamines in capsules initially presumed to contain 'ecstasy' induced the question whether these drugs will be the predominant designer drugs of the 21st century [2]. Originally engineered in the former Soviet states, these psychoactive substances diffused over central Europe to the US [3]. The emergence of 4-MMC was recently described, in contrast to the appearance of MC, which was reported somewhat earlier. Additional modifications such as fluoro derivatives are already established [4]. While in some European countries 4-MMC is currently classified as an illegal drug, in other countries it is still totally legal to buy 4-MMC, especially by internet. The consumption of MC causes several adverse effects, especially neurotoxic effects, tachycardia and convulsion, which not rarely causes the death of the abuser [1, 3, 5]. It was reported that the intake of MC causes Parkinsonism despite manganese contamination [6] and that the concomitant use of 4-MMC with heroine triggers multi-drug toxicity [7]. In contrast to its acute toxic effects [4, 8] the long-term consequences of 4-MMC consumption have been rarely investigated. MC is the beta-keto form of methamphetamine (Fig. 1). 4-MMC is structurally highly related to MC and similar negative effects are most likely.

In view of the described damaging effects caused by the intake of MC and 4-MMC, a reliable and fast quantification method is required. The control of concomitant use of these substances during a drug substitution therapy is indispensable and helps to guarantee the success of the therapy. In the critical situation of intoxication or the suspicion of an overdose, a level determination as well as a confirmation of the consumption is necessary. With a reliable quantification method the detoxification of these substances can be monitored, allowing the progress of convalescence of the patient.

MC and 4-MMC are not detected with routine amphetamine screening tests, especially immunoassays [9]. The determination by GC-MS is generally aligned to a time-consuming sample preparation and derivatization of the substances [10]. Different methods for the determination of MC in serum and urine have been described [10–13], including an accurate HPLC-MS/MS method with excellent validation statistics for the quantification by direct injection of urine [14]. By contrast, we used an additional liquid-liquid extraction technique to yield cleaner analyte extracts to minimize matrix effects and to preserve the HPLC column and the mass spectrometry instruments from contamination. Recently, some cases of 4-MMC intoxication were reported and the quantification of the serum and urinary levels was done with HPLC-tandem mass spectrometry (LC-MS/MS) [4]. However, a detailed description of the method and the validation procedure was not given.

A HPLC-MS/MS method is characterized by a fast clean-up and a short analysis time. Additionally, a LC-MS/MS method exhibits in general a very high selectivity for the analytes as well as an accurate limit of quantification. Here, we present a solid LC-MS/MS method for the quantitative determination of 4-MMC and MC in urine prepared by an unproblematic liquid-liquid extraction technique.

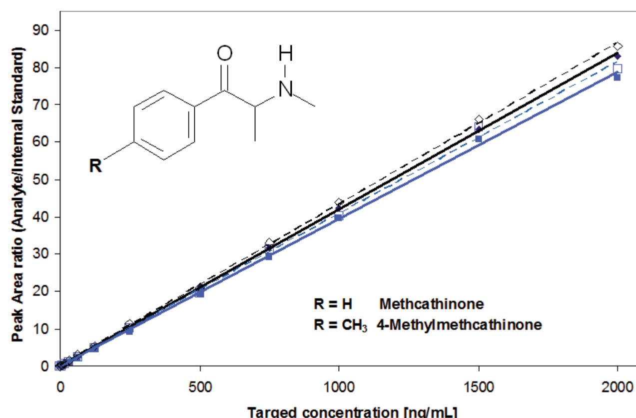


**Fig. 1.** (Color online) Chemical structures and linear behavior (1/$x$ weighted least-square regression) between 0.5 and 2000 ng/mL of MC (blue) and 4-MMC (black) obtained from the measurement of aqueous (broken line, open symbols) and urinary (urine A) samples (continuous line, filled symbols).

## 2 Materials and methods

### 2.1 Chemicals, reagents and standards

Methcathinone hydrochloride was obtained from Lipomed GmbH (Weil am Rhein, Germany), and 4-methylmethcathinone hydrochloride and the deuterated internal standard MBDB-D5 were purchased from LGC standards (Wesel, Germany). β-glucuronidase/arylsulfatase (EC 3.2.1.31/3.1.6.1) and diisopropyl ether were bought from Merck (Darmstadt, Germany). Methanol of LC-MS quality, ammonium acetate and formic acid were purchased from Sigma-Aldrich (Munich, Germany). Deionized water (18.5 MΩcm, pH 5.5) was prepared by an EASYpure RF compact ultrapure water system (Barnstead Co., USA) and it was used as a solvent or subphase in all experiments.

Standards and control solutions were made by dissolving the hydrochlorides in water to a stock concentration of 10 μg/mL of pure MC and 4-MMC, respectively. The stock solutions were added to water or the urinary matrices to obtain solutions which contained 2000 and 1500 ng/mL of both substances. Additional serial dilutions were made to achieve concentrations of 1000, 750, 500, 250, 125, 62.5, 31.3, 15.6, 7.8, 3.9, 2.0, 1.0 and 0.5 ng/mL. Therefore, 1000-μL volumes of the spiked samples were mixed with 1000 μL of water or urine to achieve the subsequent concentrations.

### 2.2 Sample and standard treatment

100 μL of the urinary samples, calibration standards and quality controls were diluted with a 300-μL solution containing the internal standard MBDB-D5 (8.4 ng/mL) and β-glucuronidase/arylsulfatase (0.1/0.2 U/mL). The samples

**Table I.** Values of the API 4000 mass spectrometer.

| Compound | M [H$^+$] | MRM transition | CE (V) | CXP (V) | DP (V) | EP (V) |
|---|---|---|---|---|---|---|
| 4-MMC | 178.100 | 159.900 | 19.0 | 12.0 | | |
| | 178.100 | 144.900 | 29.0 | 10.0 | 33.0 | 10.0 |
| | 178.100 | 118.900 | 31.0 | 8.0 | | |
| MC | 164.047 | 145.800 | 19.0 | 10.0 | | |
| | 164.047 | 130.800 | 27.0 | 10.0 | 36.0 | 10.0 |
| | 164.047 | 105.100 | 31.0 | 8.0 | | |
| MBDB-D5 | 213.140 | 135.900 | 25.0 | 10.0 | | |
| | 213.140 | 178.800 | 17.0 | 14.0 | 30.0 | 8.0 |
| | 213.140 | 78.000 | 59.0 | 8.0 | | |
| Source values | Collision gas (CAD) | Curtain gas (CUR) | Ion source gas 1 (GS1) | Ion source gas 2 (GS2) | Ion spray voltage | Temperature (°C) |
| | 11 | 15 | 55 | 40 | 3500 | 700 |

Analysis was executed in positive mode (ESI) at unit resolution.

were slightly mixed and incubated for 1 h at 40 °C (deglycosylation step). After cooling to room temperature, 10 µL of NaOH (10 M) were added and the mixture was incubated for 10 min. The liquid-liquid extraction of the analytes was carried out by adding 750 µL of diisopropyl ether and extensive shaking on a vortex mixer for 10 min. After centrifugation, 50 µL of the separated upper phase were diluted with 150 µL diisopropyl ether in the glass sample vials.

### 2.3 Immunological tests and urine analytics

Tests of capture of MC and 4-MMC by an immunological screening method were achieved with the amphetamine/methamphetamine II fluorescence polarization immunoassay (FPIA) on an AxSYM immunochemical automated analyzer (Abbott Laboratories, Wiesbaden, Germany) [9]. Creatinine values in urine were determined with an automated urine analyzer (AU 640, Olympus Europa GmbH, Hamburg, Germany) following the Jaffé reaction [15].

The specific gravities of the urinary matrices used were defined with Multistix® 10SG urine analytic test strips (Siemens Healthcare Diagnostics Inc., Tarrytown, USA).

The urines used for the confirmation of the specificity of the method were screened for opiates, amphetamines, benzodiazepines, cannabinoides, cocaine and methadone (AxSYM automated analyzer) and confirmed with different standard GC-MS methods (Shimadzu QP2010) which are not described within this article. Promethazine and its metabolites were verified by GC-MS analysis.

### 2.4 Chromatographic and spectrometric analyses

The LC-MS/MS analysis was done on an API 4000 Triple Quad (AB SCIEX) using an Agilent HPLC system (1200 Series, Agilent Technologies). Analyst Software 1.5 (AB SCIEX) was used for the control of the mass spectroscopy instrument and the HPLC system. Chromatographic separation was done on a ZORBAX Eclipse XDB-C18 column (4.6 × 150 mm, 5 µm, Agilent Technologies) with a mobile phase of 95% methanol, 4 mM ammonium formiate and 1.6 mM formic acid in deionized water (750 µL/min, isocratic).

The multiple reaction monitoring (MRM) analysis was performed using electrospray ionization (ESI) in positive mode and unit resolution (Tab. I). After an initial Q1 Scan the MRM transitions were established by direct infusion of a dilution of MC, 4-MMC and the internal standard MBDB-D5 in the mobile phase. The compound optimization procedure was applied to obtain the final ion path parameters for the different transitions. Source parameters such as ion spray voltage, temperature, curtain gas, collision gas and source gases were optimized by the application of flow injection analysis. It should be considered that the ion path parameters and the source parameters are not ubiquitous for each mass spectrometer and have to be optimized individually. The values should be transferable within the same device type.

### 2.5 Validation procedure

Method validation included studies of linearity, precision (repeatability and intermediate precision), accuracy, and the estimation of the lower limit of quantification (LOQ) and the limit of detection (LOD), as well as tests concerning the stability and the specificity of the method.

#### 2.5.1 Linearity

Linearity of a method is defined as the linear relationship between experimental test results and sample concentration [16].

To prove this relationship the peak area ratios (analyte/internal standard), which are a function of the experimental concentrations (internal standard method), were plotted against the theoretical analyte concentrations. The $y$-intercept, slope and linear correlation coefficient of the test series were determined by a $1/x$ weighted least-square linear regression analysis using the program Analyst 1.5 (AB SCIEX). The linear range was investigated up to analyte concentrations of 2000 ng/mL (0, 0.5, 1.0, 3.9, 7.8, 15.6, 31.3, 62.5, 125, 250, 500, 750, 1000, 1500 and 2000 ng/mL) for both urinary (urine A, urine B) and aqueous samples.

Matrix effects (ion suppression or enhancement) can be analyzed by the comparison of the calibration curve of pure

substance solutions with the calibrations of matrix samples with the same concentrations [17]. Matrix independence was proved by comparing the peak area ratios of the aqueous calibration curves with those of the urinary test series measured subsequently.

#### 2.5.2 Precision and accuracy

Precision can be expressed as both repeatability (intra-day precision) and intermediate precision (inter-day precision) [16]. Precision was reported by determination of the coefficients of variation (CV) of numerous inter-day and intra-day test series (urinary and aqueous samples) at different concentrations (15.6, 62.5 and 2000 ng/mL) covering the tested linear range of the method.

Accuracy was reported as percent recovery of the different test series [16]. Additional inter-matrix experiments were performed to prove the accuracy and precision over 10 different urines.

#### 2.5.3 Limit of quantification (LOQ) and limit of determination (LOD)

The LOQ (defined as the concentration below which the analytical method cannot operate with an acceptable precision [17]) was determined by the inspection of the accuracy and repeatability of small analyte concentrations. The LOD (smallest amount of analyte that can be reliably distinguished from zero) was assumed to be 1/3 LOQ [16].

#### 2.5.4 Specificity and stability

Specificity was tested by analyzing numerous beta-keto amphetamine-negative proband urines for interfering peaks at the retention times of MC and 4-MMC.

The stability was investigated by comparison of the experimental concentrations before and after 14 periods of freezing and thawing (>20 h/>1h) of aqueous and urinary control samples and after storage at room temperature for 24 h.

## 3 Results and discussion

### 3.1 Immunological tests

MC and 4-MMC are generally not captured with standard immunoassays. Representatively, we investigated spiked urinary samples with a commonly utilized amphetamine FPIA screening test (Abbott amphetamine/metamphetamine II on AxSYM) which detects besides amphetamine and methamphetamine different amphetamine derivatives such as DOM, MDA, MDE and MDMA. Both the MC and the 4-MMC spiked samples (50, 100, 200 and 500 ng/mL, respectively) were tested clearly negative.

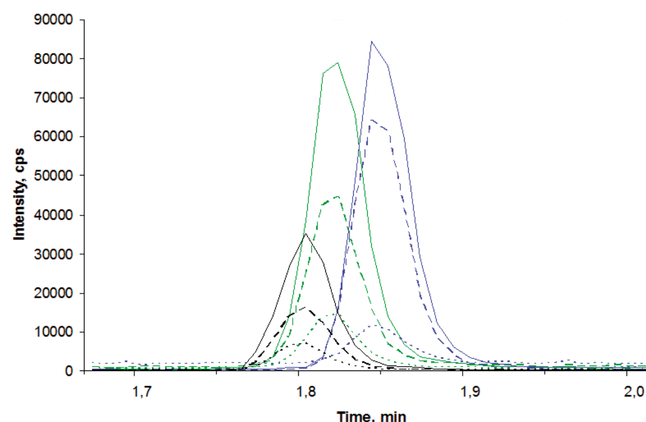


**Fig. 2.** (Color online) Chromatograms of MC (green), 4-MMC (blue) and the internal standard MBDB-D5 (black). For each substance, the three most intense mass transitions (Tab. I) are shown in solid, dashed and dotted lines.

### 3.2 Chromatographic and spectrometric analysis

An initial Q1 Scan revealed the m/z values according to the masses of the mono-protonated analytes. Tandem mass spectrometry analysis resulted for MC and 4-MMC in the three most intense product ions, which are identical to those described (Tab. I, Fig. 2) [2,4,14]. The most intense mass transition was used as a quantifier, respectively.

The method validation was confirmed on a C18 phase which provided suitable retention of the target compounds (MC, 4-MMC and the IS) and revealed symmetric peak profiles (Fig. 2). The chromatographic part of the method was optimized to isocratic conditions with a mobile methanol/water phase with respect to separation of the void volume and interfering contaminations.

### 3.3 Validation procedure

It is known that glucuronidation takes place in the metabolism of most primary, secondary and tertiary amine substrates [18]. In human metabolism at least two UDP-glucuronosyltransferase enzyme families are known (UGT1, UGT2) whereof some members have been shown to catalyze N-glucuronidation of secondary amine xenobiotics.

The metabolism of MC and 4-MMC has recently been discussed [19–21]. Besides the non-metabolized substances some metabolites (which resulted from N-demethylation, reduction of the keto moiety and, in the case of 4-MMC, from additional oxidation of the tolyl moiety) have been identified in the urine of abusers and there were some hints that primary and secondary amine metabolites of 4-MMC were partially glucuronidated or sulfonylated [20]. It remained uncertain whether these metabolites (also carrying a hydroxyl group) were N- and/or O-modified.

MC and 4-MMC are secondary amines and although there is no evidence for glucuronidation of these substances we added a deglucuronidation step by incubation of the urines with glucuronidase to capture possible glucuronosyl aggregates in LC-MS/MS analysis.

**Table II.** Regression analysis of the experimental values for the peak area ratios (analyte/internal standard) of the aqueous and urinary test series.

| Theoretical concentration (ng/mL) | MC Water | MC Urine A | MC Urine B | 4-MMC Water | 4-MMC Urine A | 4-MMC Urine B |
|---|---|---|---|---|---|---|
| 0.0 | No peak | No peak | No peak | No peak | No peak | No peak |
| 0.5 | 0.0191 | 0.0145 | 0.0164 | 0.0228 | 0.0184 | 0.0179 |
| 1.0 | 0.0344 | 0.0256 | 0.0333 | 0.0372 | 0.0378 | 0.0371 |
| 2.0 | 0.0623 | 0.0464 | 0.0715 | 0.0854 | 0.0647 | 0.0830 |
| 3.9 | 0.122 | 0.100 | 0.149 | 0.160 | 0.137 | 0.166 |
| 7.8 | 0.257 | 0.216 | 0.330 | 0.340 | 0.261 | 0.340 |
| 15.6 | 0.553 | 0.429 | 0.570 | 0.678 | 0.519 | 0.652 |
| 31.3 | 1.13 | 0.892 | 1.04 | 1.43 | 1.10 | 1.24 |
| 62.5 | 2.31 | 2.30 | 2.73 | 2.87 | 2.47 | 2.87 |
| 125 | 4.95 | 4.85 | 5.85 | 5.43 | 5.19 | 6.13 |
| 250 | 10.3 | 9.39 | 11.1 | 11.3 | 10.0 | 12.3 |
| 500 | 19.4 | 19.6 | 22.5 | 20.9 | 21.3 | 23.7 |
| 750 | 31.2 | 29.3 | 30.3 | 33.1 | 31.7 | 31.7 |
| 1000 | 40.2 | 39.8 | 42.2 | 43.9 | 42.4 | 46.3 |
| 1500 | 63.9 | 60.6 | 58.3 | 66.2 | 63.5 | 60.8 |
| 2000 | 79.6 | 77.3 | 86.3 | 85.6 | 83.0 | 92.2 |
| $f(x)$ [1] | $0.0407*x$ –0.0124 | $0.0392*x$ –0.0227 | $0.0419*x$ –0.0078 | $0.0435*x$ –0.0006 | $0.0419*x$ –0.0131 | $0.0446*x$ –0.0050 |
| Correlation coefficient ($r$) | 0.9994 | 0.9995 | 0.9986 | 0.9998 | 0.9998 | 0.9981 |

[1]Calculated with $1/x$ weighted least-square regression, $x$ = theoretical concentrations, $f(x)$ = peak area ratios (analyte/internal standard).

Glucuronidase (helix pomatia) was found to have a broad pH working range (pH 4–7) with an optimal value between pH 5 and pH 6 [22]. The pH values of the urines used (pH 5.0–7.0) were within physiological ranges, especially at pH 5.5. So we did not adjust the pH to optimal conditions.

In routine analysis heterogeneous pH values will generate fluctuations of deglucuronidation recovery and it remains questionable whether deglucuronidation is actually necessary. Anyway, by practicing deglucuronidation the pH should be adjusted, but it should be considered that this further technical step will increase the quantity of interfering substances.

Matrix effects are a ubiquitous problem in LC-MS/MS analysis that often result in ion suppression or ion enhancement [23] and therefore in quantification errors. An internal standard can compensate for these disturbing effects only partially. To reduce matrix effects in quantification of MC and 4-MMC we decided to use liquid-liquid extraction of the analytes together with the internal standard from the urinary matrix. Liquid-liquid extraction should result in clean extracts [23]. We chose for LC-MS/MS analysis an unusually long C18 column to achieve a better chromatographic separation and to reduce the co-elution of interfering matrix compounds. Finally, we decided to dilute the purified samples before injection to reduce matrix effects too, keeping in mind that our method did not lose adequate sensitivity.

### 3.3.1 Linearity

We proved the linearity of the method with aqueous solutions of the analytes as well as with two different urinary matrices (urines A and B) enriched with MC and 4-MMC.

The tested concentration range was from 0.5 to 2000 ng/mL for both analytes. Additional extraction and LC-MS/MS analysis revealed an accurate linear behavior within the tested range with correlation coefficients above 0.99 for both analytes (Fig. 1, Tab. II). A linear $1/x$ weighted least-square regression model was used for calibration. The linearity tests revealed almost the same calibration curves (theoretical concentrations *vs.* area ratios) for the spiked urinary matrices and the aqueous solutions (Fig. 1). We therefore decided to take aqueous standards for the method validation. In the following validation procedure the initially assumed matrix independence was definitely approved by comparison of the experimental recovery values of numerous urinary and aqueous test samples with the target concentrations (Tab. III). To display the diversity of the urinary matrices used we determined their creatinine values as well as their specific gravities.

### 3.3.2 Precision and accuracy

The accuracy and the precision (repeatability and intermediate precision) of the method were evaluated by means of the recovery values and the coefficients of variation (CV) of 50 different test series (Tab. III). The experimental concentration values were calculated by fitting the peak area ratios (analyte/internal standard) to the aqueous calibration curves. For validation, the intermediate precision and accuracy of 11 interday test series of aqueous solutions as well as of ten spiked urines (urines 1–10) at two different concentrations (15.6 and 62.5 ng/mL) were determined by the comparison of the experimental concentration values obtained with the target concentrations. Each test series included ten values.

**Table III.** Repeatability (CV%), intermediate precision (CV%) and accuracy (% recovery) of MC and 4-MMC obtained from solutions in water and different urinary matrices.

| Matrix(2) \ Analyte | Repeatability(1) MC | Repeatability(1) 4-MMC | Accuracy(1) (Intra-day recovery) MC | Accuracy(1) (Intra-day recovery) 4-MMC | Intermediate precision(1) MC | Intermediate precision(1) 4-MMC | Accuracy(1) (Inter-day recovery) MC | Accuracy(1) (Inter-day recovery) 4-MMC |
|---|---|---|---|---|---|---|---|---|
| $H_2O$ | 9.1/9.1 | 3.8/10.8 | 106/99 | 97/101 | 7.0/13.5 | 5.5/8.0 | 99/99 | 105/102 |
| Urine A (1.005/3.4) | 11.0/14.1 | 7.4/8.2 | 109/110 | 99/101 | 7.8(3) | 5.0(3) | 92(3) | 94(3) |
| Urine B (1.020/27.0) | 3.9/4.2 | 2.5/2.4 | 109/110 | 99/100 | 6.9(3) | 4.7(3) | 102(3) | 97(3) |
| Urine 1 (1.030/4.1) | – | – | – | – | 9.2/6.3 | 4.7/3.8 | 107/107 | 103/101 |
| Urine 2 (1.010/11.0) | – | – | – | – | 9.6/6.9 | 6.1/4.1 | 96/94 | 98/96 |
| Urine 3 (1.025/6.9) | – | – | – | – | 9.4/4.4 | 6.2/3.3 | 98/102 | 97/100 |
| Urine 4 (1.025/6.9) | – | – | – | – | 8.7/8.8 | 5.2/4.4 | 105/98 | 100/96 |
| Urine 5 (>1.030/13.4) | – | – | – | – | 8.5/10.0 | 4.2/5.6 | 116/115 | 108/105 |
| Urine 6 (> 1.030/4.7) | – | – | – | – | 6.8/11.0 | 4.6/6.4 | 116/117 | 109/108 |
| Urine 7 (1.020/3.7) | – | – | – | – | 8.1/10.3 | 5.0/5.5 | 105/107 | 105/106 |
| Urine 8 (>1.030/3.5) | – | – | – | – | 9.4/8.5 | 4.4/4.4 | 116/111 | 107/103 |
| Urine 9 (1.030/5.8) | – | – | – | – | 8.5/9.4 | 4.2/4.2 | 97/100 | 95/99 |
| Urine 10 (1.025/3.6) | – | – | – | – | 8.8/11.4 | 4.8/7.5 | 103/103 | 103/99 |

| | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 | Day 6 | Day 7 | Day 8 | Day 9 | Day 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| Inter-matrix precision(4) MC | 12.6/10.9 | 9.3/7.1 | 11.2/10.3 | 8.7/9.4 | 9.0/6.0 | 12.0/10.3 | 11.1/10.2 | 10.0/10.9 | 10.0/9.0 | 10.3/6.3 |
| Inter-matrix precision(4) 4-MMC | 6.4/3.9 | 7.0/4.9 | 5.5/5.3 | 4.9/5.9 | 5.4/4.5 | 4.9/6.8 | 6.7/7.1 | 6.5/7.1 | 7.2/4.9 | 7.4/3.8 |
| Accuracy(4) (Inter-matrix) MC | 104/100 | 102/101 | 109/114 | 114/111 | 108/109 | 105/106 | 103/102 | 110/112 | 98/90 | 108/109 |
| Accuracy(4) (Inter-matrix) 4-MMC | 100/98 | 102/103 | 102/105 | 109/105 | 103/102 | 101/102 | 100/97 | 105/104 | 100/95 | 104/104 |

(1) The tested concentrations of MC and 4-MMC were 15.6 ng/mL and 62.5 ng/mL. The values for each concentration are separated by a slash
(2) Specific gravity and creatinine values [mM] are given in parenthesis and are separated by a slash.
(3) Tested analyte concentration was 2000 ng/mL.
(4) The values refer to the intra-day measurements across ten different matrices (urine 1–urine 10) within one series.

We additionally monitored the intra-day counterparts of two different urines (urine A, urine B) and of aqueous solutions at both concentrations. To prove whether the method continued to measure with a sufficient accuracy at upper concentrations the intermediate precision and recovery were also determined at 2000 ng/mL for the two different urines. Finally, we calculated some inter-matrix values across ten different urinary matrices (urines 1–10) measured within one series at concentrations of 15.6 and 62.5 ng/mL.

Overall, in 50 different test series (24 for 15.6 and 62.5 ng/mL, respectively; two for 2000 ng/mL) the accuracy for 4-MMC was within 9% and for MC within 17% of the target concentration (Tab. III). The intermediate precision and the repeatability were between 4 and 14% for MC and between 2 and 8% for 4-MMC. Between the inter-day and inter-matrix values no remarkable differences could be detected. The calculated concentrations of the urinary calibration curves (urine A, urine B) analyzed at the beginning of the validation procedure

also fitted well with the target concentrations. The accuracy and intermediate precision obtained at 2000 ng/mL (Tab. III) proved the method to be practicable up to the upper concentration limit of the tested linearity range.

#### 3.3.3 Limit of quantification (LOQ) and limit of detection (LOD)

The lower limit of quantification is the concentration where the determination of the analyte is possible with reasonable statistical certainty. We used two different spiked urine matrices (urines A and B, Tab. III) at three different analyte concentrations (MC: 2.0, 3.0 and 5.0 ng/mL; 4-MMC: 0.8, 1.0 and 1.5 ng/mL). Each sample was extracted and analyzed ten times to determine the repeatability and accuracy. Acceptable coefficients of variation (CV) and recoveries were obtained at 0.8 ng/mL for 4-MMC (urine A: 5.3/110%, urine B: 9.9/98%) and at 2.0 ng/mL for MC (urine A: 8.4/113%, urine B: 9.9/113%). The lower limit of detection can be assumed to be one-third of the LOQ [16]. The upper limit of quantification was established to be 2000 ng/mL, with acceptable intermediate precision and accuracy (Tab. III).

#### 3.3.4 Specificity and stability

To confirm the specificity of our method we analyzed 29 different MC- and 4-MMC-negative urines. Nineteen urines were obtained from drug abusers and were tested highly positive for amphetamine, cocaine and its metabolites methylecgonine and benzoylecgonine, opiates (morphine, codeine, 6-monoacetylmorphine), promethazine, norpromethazine and norpromethazine-sulfoxide, benzodiazepines (oxazepam, nordazepam, diazepam, temazepam, midazolam, 7-aminoflunitrazepam), cannabinoids (THC-COOH), methadone and EDDP.

None of the tested extracts showed interfering peaks at the retention time of MC or 4-MMC, which proves the specificity of the method.

Daily freezing and thawing of the aqueous and the urinary control samples over a period of more than 14 days as well as leaving at room temperature for 24 h did not alter the analyte concentrations.

## 4 Conclusions

We generated and validated a stable quantification method for the determination of the beta-keto amphetamines MC and 4-MMC in urine.

The sample preparation was based on an easy liquid-liquid extraction procedure with reversed phase chromatography. Coupled with the MRM tandem mass spectrometry technique no interfering influence of the matrix in quantification was observed and a high specificity for the analytes could be achieved.

The method was shown to be linear over a broad concentration range and exhibited a low limit of detection and limit of quantification, which enables the determination of trace levels.

The analytes were proved to be stable in the utilized urinary analysis samples and the aqueous calibration and control materials.

The achieved validation statistics are acceptable for clinical routine analysis. Because there are no immunological screening methods, the presented method is helpful for the supervision of drug substitution therapy and important for clarification of intoxication. The method should be expandable with additional beta-keto amphetamines such as fluoro derivatives of 4-MMC.

**Conflict of interest.** The authors declare that there are no conflicts of interest.

## References

1. Belhadj-Tahar H, Sadeg N. Methcathinone: a new postindustrial drug. Forensic Sci Int. 2005; 153: 99-101.
2. Roussel O, Perrin M, Herard P, Chevance M, Arpino P. Is 4-methylephedrone, an “Ecstasy” of the twenty first century? Ann Toxicol Anal. 2009; 21: 169-177.
3. Emerson TS, Cisek JE. Methcathinone: a Russian designer amphetamine infiltrates the rural midwest. Ann Emerg Med. 1993; 22: 1897-1903.
4. Davies S, Puchnarewicz M, Button J, Dargan PI, Wood DM, Archer R, Ramsey J, Lee T, Holt DW. Two cases of confirmed ingestion of the novel designer compounds: 4-methylmethcathinone (mephedrone) and 3-fluoromethcathinone. Ann Toxicol Anal. 2009; 21: S1.
5. Sparago M, Wlos J, Yuan J, Hatzidimitriou G, Tolliver J, Dal Cason TA, Katz J, Ricaurte G. Neurotoxic and pharmacologic studies on enantiomers of the N-methylated analog of cathinone (methcathinone): a new drug of abuse. J Pharmacol Exp Ther. 1996; 279: 1043-1052.
6. Stephens A, Logina I, Liguts V, Aldins P, Eksteina I, Platkajis A, Martinsone I, Terauds E, Rozentale B, Donaghy M. A Parkinson syndrome in methcathinone Users and the role of manganese. N Engl J Med. 2008; 358: 1009-1017.
7. Dickson AJ, Vorce SP, Levine B, Past MR. Multiple-drug toxicity caused by the coadministration of 4-methylmethcathinone (mephedrone) and heroin. J Anal Toxicol. 2010; 34: 162-168.
8. Wood DM, Davies S, Puchnarewicz M, Button J, Archer R, Ovaska H, Ramsey J, Lee T, Holt DW, Dargan PI. Recreational use of mephedrone (4-methylmethcathinone, 4-MMC) with associated sympathomimetic toxicity. J Med Toxicol. 2010; 6: 327-330.
9. Verstraete AG, Heyden FV. Comparison of the sensitivity and specificity of six immunoassays for the detection of amphetamines in urine. J Anal toxicol. 2005; 5: 359-364.
10. Valentine JL, Middleton R. GC-MS identification of sympathomimetic amine drugs in urine: rapid methology applicable for emergency clinical toxicology. J Anal Toxicol. 2000; 24: 211-222.
11. Liau AS, Liu LC, Chiu YC, Shu YR, Tsai CC, Lin CH. Optimization of a simple method for the chiral separation of methamphetamine and related compounds in clandestine tablets and urine samples by beta-cyclodextrine modified capillary electrophoresis: a complementary method to GC-MS. Forensic Sci Int. 2003; 134: 17-24.
12. Wohlfarth A, Weinmann W, Dresen S. LC-MS/MS screening method for designer amphetamines, tryptamines, and piperazines in serum. Anal Bioanal Chem. 2010; 396: 2403-2414.
13. Beyer J, Peters FT, Kraemer T, Maurer HH. Detection and validated quantification of nine herbal phenalkylamines and methcathinone in human blood plasma by LC-MS/MS with electrospray ionization. J Mass Spectrom. 2007; 42: 150-160.

14. Thevis M, Sigmund G, Thomas A, Gougoulidis V, Rodchenkov G, Schänzer W. Doping control analysis of metamfepramone and two major metabolites using liquid chromatography-tandem mass spectrometry. Eur J Mass Spectrom. 2009; 15: 507-515.
15. Seelig HP. The Jaffé reaction with creatinine. The reaction product and general reaction conditions. Z klin Chem klin Biochem. 1969; 7: 581-585.
16. International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. ICH Harmonised Tripartite Guideline Q2(R1): Validation of analytical procedures: text and methodology. Geneva, Switzerland, October 27, 1994.
17. Thompson M, Ellison SLR, Wood R. Harmonized guidelines for single-laboratory validation of methods of analysis (IUPAC technical report). Pure Appl Chem. 2002; 74: 835-855.
18. Green MD, Tephly TR. 1996 ASPET N-Glucuronidation of Xenobiotics Symposium. Glucuronidation of Amine substrates by purified and expressed UDP-glucuronosyltransferase proteins. Drug Metab Dispos. 1998; 26: 860-867.
19. Markantonis SL, Kyroudis A, Beckett AH. The stereoselective Metabolism of dimethylpropion and monomethylpropion. Biochem Pharmacol. 1986; 35: 529-532.
20. Meyer MR, Wilhelm J, Peters FT, Maurer HH. Beta-keto amphetamines: studies on the metabolism of the designer drug mephedrone and toxicological detection of mephedrone, butylone, and methylone in urine using gas chromatography-mass spectrometry. Anal Bioanal Chem. 2010; 397: 1225-1233.
21. Meyer MR, Maurer HH. Metabolism of designer drugs of abuse: an updated review. Curr Drug Metab. 2010; 11: 468-482.
22. Le Bizec B, Montrade MP, Monteau F, Andre F. Detection and identification of anabolic steroids in bovine urine by gas chromatography-mass spectrometry. Anal Chim Acta. 1993; 275: 123-133.
23. Chambers E, Wagrowski-Diehl DM, Lu Z, Mazzeo JR. Systematic and comprehensive strategy for reducing matrix effects in LC/MS/MS analyses. J Chromatogr B. 2007; 852: 22-34.